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UTILITY PATENT APPLICATION TRANSMITTAL AND FEE SHEET

Transmitted herewith for filing under 37 CFR §1.53(b)(1) is a **continuation** of prior Application No. 09/159,027, filed September 23, 1998.

Applicant (or identifier): GRONER ET AL.

Title: BIFUNCTIONAL PROTEIN, PREPARATION AND USE

Enclosed are:

1. ☒ Specification (Including Claims and Abstract) - 39 pages
 2. ☒ Drawings - 1 sheets
 3. Declaration and Power of Attorney
 - a. ☐ Newly executed (original or copy)
 - b. ☒ Copy from a prior application (signed or with indication that original was signed)
 - i. ☐ Deletion of Inventors
Signed statement attached deleting inventor(s) named in the prior application
 4. ☒ Incorporation By Reference
The entire disclosure of the prior application, from which a copy of the Declaration and Power of Attorney is supplied under Box 3b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
 5. ☐ Microfiche Computer Program (appendix)
 6. Nucleotide and/or Amino Acid Sequence Submission
 - ☐ Computer Readable Copy
 - ☐ Paper Copy
 - ☐ Statement Verifying Identity of Above Copies
 7. ☒ Preliminary Amendment
 8. ☐ Assignment Papers (Cover Sheet & Document(s))
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 10. ☐ Information Disclosure Statement
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 12. ☒ Return Receipt Postcard
 13. ☐ Other:
- ☒ The right to elect an invention or species that is different from that elected in parent Application No. 09/159,027 in the event of a restriction or election of species requirement that is identical or substantially similar to that made in said parent application is hereby reserved.

The PRO did not receive the following listed item(s):
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Filing fee calculation:

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Basic Filing Fee							\$	690
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Extra Claims	Total Claims	15	-20	0	x	\$	18	= \$
	Independent Claims	1	-3	0	x	\$	78	= \$
TOTAL FILING FEE							\$	690

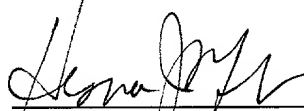
- ☒ Please charge Deposit Account No. 19-0134 in the name of Novartis Corporation in the amount of \$690. An additional copy of this paper is enclosed. The Commissioner is hereby authorized to charge any additional fees under 37 CFR §1.16 and §1.17 which may be required in connection with this application, or credit any overpayment, to Deposit Account No. 19-0134 in the name of Novartis Corporation.

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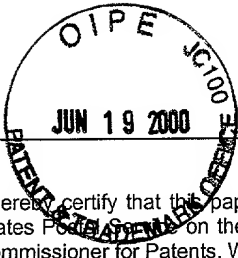
Respectfully submitted,



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Date: 6/19/00

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CASE 4-19924C/C1C1

CERTIFICATE OF MAILING

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF

Art Unit: 1642

GRONER ET AL.

Examiner: UNGAR, SUSAN

APPLICATION NO: UNKNOWN

FILED:

FOR: BIFUNCTIONAL PROTEIN, PREPARATION AND USE

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to calculation of the above-mentioned application's filing fees, please amend the claims as follows:

IN THE CLAIMS:

Claim 4, line 1, delete "or 3".

Claim 5, line 1, delete "to 4".

Claim 6, line 1, delete "any of claims 2 to 5" and replace with -- claim 2 --.

Claim 9, lines 2 and 3, delete "claims 2 to 4" and replace with -- claim 2 --.

Claim 10, line 2, delete "of claim 6" and replace with -- of containing DNA encoding said protein --.

Claim 11, line 1, delete "claims 6 or 7" and replace with -- claim 7 --.

Claim 15, line 1, delete "claims 2 to 5" and replace with -- claim 2 --.

IN THE SPECIFICATION:

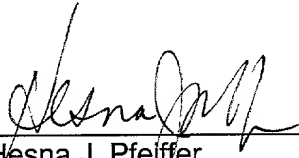
P.1, add this sentence as the first sentence: "This application is a continuation of USSN 09/159,027 filed September 23, 1998, now _____, which was a continuation of USSN 08/703,048, filed 01.11.96 now abandoned, from PCT National filing of PCT/EP95/01494 filed

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20.04.95 filed under PCT Article 34 on 13.03.96, and which claims foreign priority benefits under 35 USC 119 of EPO 94810244.7 filed 02.05.94."

Respectfully submitted,

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Bifunctional Protein, Preparation and Use

The present invention relates to a bifunctional protein capable of directing a host cell producing said protein to specifically recognize selected target cells. Furthermore, the invention provides a method for the preparation of said protein, a DNA construct encoding said protein, a composition comprising a host cell expressing said DNA, and antibodies specifically recognizing said protein. Additionally, the invention relates to the use of such a host cell, e.g. for selectively killing tumor cells in vitro or in vivo.

The principle of adoptive immunotherapy, also referred to as cellular immunotherapy, is the transfer of immunologically active cells to a mammal in order to enhance the mammal's immune response to a disease state. To this end the immune cells are removed e.g. from the human patient or another subject, cultured, optionally in the presence of immunoenhancing agents such as interleukin 2, and subsequently (re-)administered to the patient, conventionally in the presence of an immunoenhancing agent. In the patient, the immunologically active cells act to alleviate the disease state.

Immunologically active cells suggested for adoptive immunotherapy include lymphokine activated killer (LAK) cells, derived from natural killer (NK) cells, and in vitro sensitized lymphocytes (IVS), derived from cytolytic or cytotoxic T lymphocytes (CTL), also referred to as killer T lymphocytes. LAK cells are cytolytic cells which react with a broad spectrum of target cells. They are not major histocompatibility complex (MHC)-restricted and capable of lysing tumor cells, but also normal cells in vitro. CTL have clonal specificities, i.e. each clone is specific for a particular antigenic structure on the surface of a target cell. A particular CTL recognizes and binds a unique antigen and thus becomes activated and can then multiply and destroy the target cells. The recognition process is MHC-restricted and dependent, since an antigen is recognized only in association with one of the self class I MHC surface molecules expressed by the target cell.

Recognition of a specific antigen by T cells is mediated by the T-cell antigen receptor (TCR) (A. Weiss, Cell 73, 209-212 (1993)). Binding of a ligand to the receptor may trigger cellular effector programs, such as activation of tyrosine kinases, intracellular calcium ion release and interleukin 2 production (R.T. Abraham et al., Trends Biochem. Sci. 17, 434-438 (1992)).

The TCR is a multimeric surface complex comprising the products of at least six genes, all

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of which are required for efficient plasma membrane expression. The clonotypic alpha (α) and beta (β) chains of the TCR mediate specific target cell recognition. These chains are non-covalently associated with the non-polymorphic components of the CD3 complex gamma (γ), delta (δ), and epsilon (ϵ), and the zeta (ζ) chain. The disulfid-linked ζ homodimer is a transmembrane molecule and its cytoplasmic part plays a central role in the TCR-mediated signal transduction and induction of cytotoxicity. The ζ chain is capable of autonomous signal transduction, i.e. ζ alone is sufficient to mediate a response. Fusion of the ζ chain with an extracellular ligand binding domain may result in a molecule which can be activated by interaction with the ligand (S.J. Frank et al., Science 249, 174-177 (1990); C. Romeo & B. Seed, Cell 64, 1037-1046 (1990); F. Letourneur & R.D. Klausner, Proc. Natl. Acad. Sci. USA 88, 8905-8909 (1991)). An isoform of ζ , eta (η), represents an alternatively spliced form of the ζ gene transcript.

Tumor formation involves the mutation of oncogenes and tumor suppressor genes in somatic cells. Such mutations may result in structural alterations or in the overexpression of proteins. Both events might lead to alterations in the intracellular processing of these proteins and the presentation of new antigenic structures in association with the major histocompatibility antigens on the surface of the cells. The detection of antibodies directed against oncogene products in the serum of tumor patients is an indication that oncogene products can be antigenic. Further evidence for this antigenicity is the evocation of the cellular immune response. The occurrence of CTL which recognize and eliminate tumor cells has been demonstrated in a number of model systems (T. Boon, Adv. Cancer Res. 58, 177-210 (1992); M.W. Kast et al., Cell 59, 603-614 (1989); Disis et al., Cancer Res. 54, 16-20 (1994)).

Present strategies aimed at exploiting the cytolytic activity of T-lymphocytes, e.g. for the treatment of cancer, suffer from several shortcomings, such as MHC-restriction of the recognition process in naturally occurring CTL. There is a need for an approach to overcome the limitations currently encountered.

It is the object of the present invention to provide such an improved approach involving manipulation of CTL-recognition specificity, e.g. to make the altered CTL potent and selective anti-tumor agents. This approach is based on the identification of consistent genetic alterations in benign and particularly in malignant tumor cells. Providing CTL with a defined tumor cell specificity enables the targeting to defined tumor cells and MHC-unrestricted and MHC-independent destruction of said target cells. Tumor cell lysis

by CTL grafted with a novel, MHC independent recognition specificity may be exploited in vitro (ex vivo) or in vivo, e.g. in a gene therapy approach involving cancer treatment.

The tumor cells are (pre-)defined or selected target cells in that they carry the antigenic structure (ligand) recognized and bound by the antigen binding domain which is part of the chimeric protein of the invention.

The present invention concerns a chimeric protein capable of directing a CTL to specifically recognize and kill selected tumor cells. More specifically, the present invention provides a chimeric protein comprising a recognition function, a hinge region and the ζ chain of the TCR, and a CTL producing one or more of such protein molecules. Binding of a cell-bound ligand to the recognition part of the chimeric protein of the invention leads to ζ chain-mediated signal transduction within the CTL and eventually results in the lysis of the cell carrying the ligand.

The chimeric protein of the invention is a protein which does not exist in nature. The protein is bifunctional in that it is capable of both specifically recognizing and binding to a particular antigenic structure (via its recognition function domain) and serving as a signalling component (via the ζ chain part). The hinge region serves as a spacer and ensures the necessary accessibility and flexibility of the recognition function domain. The hinge region is understood to be essential for the functionality of the chimeric protein of the invention. Preferably, the arrangement within the chimeric protein is such that the recognition function is located at the N-terminus and linked to the ζ chain part at the C-terminus of the chimeric protein via the hinge region. Being a cell surface receptor molecule the chimeric protein of the invention comprises an extracellular domain, a transmembrane domain and a cytoplasmic domain and is inserted into the plasma membrane of the host cell, e.g. the CTL. The functionality of the protein of the invention within the host cell is detectable in an assay suitable for demonstrating the signalling potential of said protein upon binding of a particular ligand, e.g. in an assay enabling detection of a signalling pathway triggered upon binding of the ligand, such as an assay involving measurement of the increase of calcium ion release, intracellular tyrosine phosphorylation, inositol phosphate turnover or interleukin (IL) 2, interferon γ , GM-CSF, IL-3, IL-4 production thus effected. (R.T. Abraham et al., *Trend Biochem. Sci.* 17, 434-438 (1992)). Such assays are readily available to the person with ordinary skill in the art. Reference is made to the assays employed in the Examples. It is evident that these assays may be modified, e.g. by using other suitable cell lines.

The recognition function is contributed by an antigen binding domain of an antibody, particularly a single chain antibody (scFv). Single chain antibodies are gene fusions comprising the variable domains of the heavy and light chain of monoclonal antibodies. Said recognition and binding function is conferred to the ζ -chain of the TCR-complex to circumvent MHC-restricted antigen recognition through the α/β chains of the TCR.

The antigen binding domain is derivable from a monoclonal antibody directed against and specific for a suitable antigen on a tumor cell.

A suitable antigen is an antigen with enhanced or specific expression on the surface of a tumor cell as compared to a normal cell, e.g. an antigen evolving from consistent genetic alterations in tumor cells. Examples of suitable antigens include ductal-epithelial mucine, gp 36, TAG-72, growth factor receptors and glycosphingolipids and other carbohydrate antigens preferentially expressed in tumor cells (Please give references for the below captioned antigens and antibodies). Ductal-epithelial mucine is enhancedly expressed on breast, ovarian and pancreas carcinoma cells and is recognized e.g. by monoclonal antibody SM3 (Zotter et al., Cancer Rev. 11, 55-101 (1988)). The glycoprotein gp 36 is found on the surface of human leukemia and lymphoma cells. An exemplary antibody recognizing said antigen is SN 10. TAG-72 is a pancarcinoma antigen recognized by monoclonal antibody CC49 (Longenecker, Sem. Cancer Biol. 2, 355-356). Growth factor receptors are e.g. the human epidermal growth factor (EGF) receptor (Khazaie et al., Cancer and Metastasis Rev. 12, 255-274 (1993)) and HER2, also referred to as erbB-2 or gp 185 (A. Ullrich and J. Schlessinger, Cell 61, 203-212 (1990)). The erbB-2 receptor is a transmembrane molecule which is overexpressed in a high percentage of human carcinomas (N.E. Hynes, Sem. in Cancer Biol. 4, 19-26 (1993)). Expression of erbB-2 in normal adult tissue is low. This difference in expression identifies the erbB-2 receptor as "tumor enhanced".

Preferably, the antigen binding domain is obtainable from a monoclonal antibody produced by using as immunogen viable human tumor cells presenting the antigen in its native form. In a preferred embodiment of the invention, the recognition part of the chimeric protein specifically binds to an antigenic determinant on the extracellular domain of a growth factor receptor, particularly HER 2. Monoclonal antibodies directed to the HER2 growth factor receptor are known and are described, for example, by S.J. Mc Kenzie et al., Oncogene 4, 543-548 (1990), R.M. Hudziak et al., Molecular and

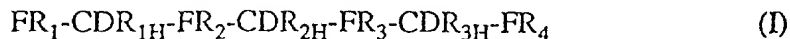
Cellular Biology 9, 1165-1172 (1989), International Patent Application WO 89/06692 (Genentech) and Japanese Patent Application Kokai 02-150 293 (Ajinomoto KK). Monoclonal antibodies raised against viable human tumor cells presenting HER2 in its native form, such as SKBR3 cells, are described, for example, in European patent application EP-A-502 812 (Ciba-Geigy) which is enclosed herein by reference, and include antibodies FRP5, FSP16, FSP77 and FWP51. Hybridoma cell lines producing these antibodies have been deposited with the European Collection of Animal Cell Cultures (ECACC, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, UK) on November 21, 1990 under accession numbers 90112115, 90112116, 90112117 and 90112118, respectively.

In the chimeric protein of the invention, the preferred antigen binding domain is a single-chain recombinant antibody (scFv) comprising the light chain variable domain (V_L) bridged to the heavy chain variable domain (V_H) via a flexible linker (spacer), preferably a peptide. Advantageously, the peptide consists of about 10 to about 30 amino acids, particularly naturally occurring amino acids, e.g. about 15 naturally occurring amino acids. Preferred is a peptide consisting of amino acids selected from L-glycine and L-serine, in particular the 15 amino acid peptide consisting of three repetitive units of Gly-Gly-Gly-Gly-Ser. Advantageous is a single-chain antibody wherein V_H is located at the N-terminus of the recombinant antibody. Preferred is a chimeric protein wherein the single-chain recombinant antibody has an above-defined preferred specificity, e.g. a chimeric protein comprising a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derivable from a monoclonal antibody, e.g. a murine monoclonal antibody, directed to the human growth factor receptor HER2, such as a murine monoclonal antibody selected from the group consisting of FSP16, FSP77, FRP5 and FWP51.

The variable domain of an antibody heavy or light chain consists of so-called framework regions (FRs), which are fairly conserved in antibodies with different specificities, and of hypervariable regions also called complementarity determining regions (CDRs), which are typical for a particular specificity. In the antigen binding domain of a chimeric protein according to the invention, preferably the FRs are derivable from a mammalian, e.g. a murine or particularly a human antibody. The scFv derivative of a monoclonal antibody is grafted onto the ζ chain of the TCR/CD3 complex.

Particularly preferred is a chimeric protein comprising a single-chain recombinant

antibody wherein the heavy chain variable domain comprises a polypeptide of the formula



wherein the polypeptide chain is described as starting at the N-terminal extremity and ending at the C-terminal extremity and FR₁ is a peptide residue comprising at least 25-29, preferably 25-33 naturally occurring amino acids, FR₂ is a peptide residue comprising 12-16 naturally occurring amino acids, FR₃ is a peptide residue comprising 30-34 naturally occurring amino acids, FR₄ is a peptide residue comprising at least 6-10, preferably 6-13 naturally occurring amino acids, CDR_{1H} is a peptide residue of the amino acid sequence 31 to 35 of SEQ ID NO:2, CDR_{2H} is a peptide residue of the amino acid sequence 50 to 66 of SEQ ID NO:2, and CDR_{3H} is a peptide residue of the amino acid sequence 99 to 108 of SEQ ID NO:2, or, CDR_{1H} is a peptide residue of the amino acid sequence 31 to 35 of SEQ ID NO:4, CDR_{2H} is a peptide residue of the amino acid sequence 50 to 66 of SEQ ID NO:4, and CDR_{3H} is a peptide residue of the amino acid sequence 99 to 109 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges. These particular complementarity determining regions are Asn-Tyr-Gly-Met-Asn (CDR_{1H}), Trp-Ile-Asn-Thr-Ser-Thr-Gly-Glu-Ser-Thr-Phe-Ala-Asp-Asp-Phe-Lys-Gly (CDR_{2H}), and Trp-Glu-Val-Tyr-His-Gly-Tyr-Val-Pro-Tyr (CDR_{3H}) according to SEQ ID NO:2, or Ser-Tyr-Trp-Met-Asn (CDR_{1H}), Met-Ile-Asp-Pro-Ser-Asp-Ser-Glu-Thr-Gln-Tyr-Asn-Gln-Met-Phe-Lys-Asp (CDR_{2H}) and Gly-Gly-Ala-Ser-Gly-Asp-Trp-Tyr-Phe-Asp-Val (CDR_{3H}) according to SEQ. ID NO:4.

Especially preferred is a chimeric protein wherein the recombinant single-chain antibody comprises a heavy chain variable domain of formula I, wherein the framework regions FR₁, FR₂, FR₃ and FR₄ are those preferably derivable from a mammalian, especially a murine or a human antibody.

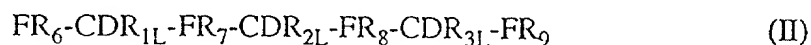
In a first embodiment of the invention, most preferred is a chimeric protein wherein the heavy chain variable domain of the recombinant single-chain antibody comprises a polypeptide of the amino acid sequence 2 to 120, of SEQ ID NO:2, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 2 to 30 (FR₁), 36 to 49 (FR₂), 67 to 98 (FR₃), and/or 110 to 120 (FR₄), are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular a chimeric protein wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 6 to 119 of SEQ ID NO:2,

wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

In a second embodiment of the invention, most preferred is a chimeric protein wherein the heavy chain variable domain of the recombinant single-chain antibody comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein optionally one or more, e.g. 1, 2, 3 or 4, amino acids within the amino acid sequences 2 to 30 (FR₁), 36 to 49 (FR₂), 67 to 98 (FR₃), and/or 110 to 120 (FR₄), are replaced with other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 6 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

For example, a hydrophobic amino acid within a framework region may be replaced by another amino acid, preferably also a hydrophobic amino acid, e.g. a homologous amino acid, replaced with two amino acids (resulting in the insertion of an amino acid), or deleted. Likewise, a hydrophilic amino acid within a framework region may be replaced with another amino acid, two amino acids or deleted, whereby replacing amino acids preferably maintain the hydrogen bond structure of the corresponding framework region. Advantageously, any replacement of one or more amino acids takes into account the guidelines known in the art for reshaping or humanizing of an antibody. Particularly noteworthy are guidelines aimed at reducing the immunogenicity of the reshaped antibody (as compared to the "original" monoclonal antibody) and/or at designing an antibody which about equals or exceeds the binding affinity of the "original" antibody. A modification of amino acids may be confined to a single FR, i.e. FR₁, FR₂, FR₃ or FR₄, or involve two, three or all four of the FRs.

A likewise preferred chimeric protein of the invention comprises a recombinant single-chain antibody wherein the light chain variable domain comprises a polypeptide of the formula



wherein the polypeptide chain is described as starting at the N-terminal extremity and ending at the C-terminal extremity and FR₆ is a peptide residue comprising naturally occurring amino acids, preferably 19-25, especially 19-23 naturally occurring amino acids, FR₇ is a peptide residue comprising 13-17 naturally occurring amino acids, FR₈ is a

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peptide residue comprising 30-34 naturally occurring amino acids, FR₉ is a peptide residue comprising naturally occurring amino acids, particularly 7-11 naturally occurring amino acids, and CDR_{1L} is a peptide residue of the amino acid sequence 158 to 168 of SEQ ID NO:2, CDR_{2L} is a peptide residue of the amino acid sequence 184 to 190 of SEQ ID NO:2, and CDR_{3L} is a peptide residue of the amino acid sequence 223 to 231 of SEQ ID NO:2, or CDR_{1L} is a peptide residue of the amino acid sequence 159 to 164 of SEQ ID NO:4, CDR_{2L} is a peptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4, and CDR_{3L} is a peptide residue of the amino acid sequence 224 to 231 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges. These particular complementarity determining regions are Lys-Ala-Ser-Gln-Asp-Val-Tyr-Asn-Ala-Val-Ala (CDR_{1L}), Ser-Ala-Ser-Ser-Arg-Tyr-Thr (CDR_{2L}), and Gln-Gln-His-Phe-Arg-Thr-Pro-Phe-Thr (CDR_{3L}) according to SEQ ID NO:2, or Lys-Ala-Ser-Gln-Asp-Ile-Lys-Lys-Tyr-Ile-Ala (CDR_{1L}), Tyr-Thr-Ser-Val-Leu-Gln-Pro (CDR_{2L}) and Leu-His-Tyr-Asp-Tyr-Leu-Tyr-Thr (CDR_{3L}) according to SEQ ID NO:4.

Especially preferred is a chimeric protein wherein the recombinant antibody comprises a light chain variable domain of formula II, wherein the peptide residues of the framework regions FR₅, FR₆, FR₇ and FR₈ are those derivable from a mammalian, especially a murine or a human, antibody.

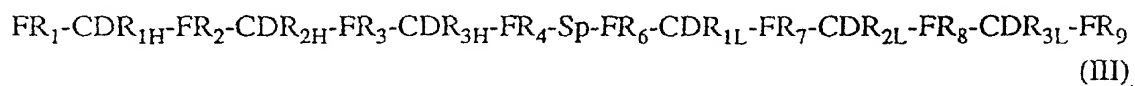
In one embodiment of the invention, most preferred is a chimeric protein wherein the recombinant antibody comprises a light chain variable domain comprising a polypeptide of the amino acid sequence 135 to 240 of SEQ ID NO:2, wherein optionally one or more, e.g. 1, 2, 3 or 4, amino acids within the amino acid sequences 135 to 157 (FR₆), 169 to 183 (FR₇), 191 to 222 (FR₈), and/or 232 to 240 (FR₉) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular a light chain variable domain comprising a polypeptide of the amino acid sequence 135 to 240 of SEQ ID NO:2, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

In a second embodiment of the invention, most preferred is a chimeric protein wherein the recombinant antibody comprises a light chain variable domain comprising a polypeptide of the amino acid sequence 136 to 240 of SEQ ID NO:4, wherein optionally one or more, e.g. 1, 2, 3 or 4 single amino acids within the amino acid sequences 136 to 158 (FR₆), 170 to 184 (FR₇), 192 to 223 (FR₈), and/or 232 to 240 (FR₉) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming

S-S-bridges, in particular a light chain variable domain comprising a polypeptide of the amino acid sequence 136 to 240 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

For example, amino acids within the framework regions may be replaced by other amino acids or deleted as detailed above for the heavy chain.

Especially preferred is a chimeric protein comprising a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group consisting of 10 to 30, e.g. about 15, amino acids, in particular a single-chain recombinant antibody comprising a polypeptide of the formula



wherein the polypeptide chain is described as starting at the N-terminal extremity and ending at the C-terminal extremity and FR₁, CDR_{1H}, FR₂, CDR_{2H}, FR₃, CDR_{3H}, FR₄, FR₆, CDR_{1L}, FR₇, CDR_{2L}, FR₈, CDR_{3L} and FR₉ have the meanings as mentioned before and Sp is a peptide spacer as disclosed above.

The antigen binding domain may be tested for its specificity to a predefined tumor cell antigen by methods known in the art, for example by immunofluorescent staining of cells expressing high levels of the antigen, by immunoblotting either directly or by way of immunoprecipitation and protein blotting of the immunocomplexes, or by another immunoassay such as binding, crossinhibition or competition radio- or enzyme immunoassay. The binding affinity of the antigen binding domain may be determined using a suitable quantitative assay which can easily be established by a person with ordinary skill in the art based on known techniques and principles. If desired, the affinity of the antigen binding domain may be compared to the affinity of a suitable reference antibody, e.g. the "parental" monoclonal mouse antibody it is derivable from.

Additionally to the antigen binding domain the chimeric protein of the invention comprises a hinge region which is inserted as a short, flexible tether between the antigen binding domain and the ζ domain. The hinge region is a peptide comprising from about 40 to about 200 naturally occurring amino acids, preferably from about 60 to about 190 amino acids. Preferably, the hinge region in the chimeric protein according to the

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invention is an immunoglobulin-like hinge region, e.g. a hinge region derivable from the CD4 molecule, such as the D3D4 immunoglobulin domains (P.J. Maddon et al., Proc. Natl. Acad. Sci. USA. 84, 9155-9159 (1987)) or a hinge region derivable from the CD8 α molecule, e.g. L γ t-2 (R. Zomoyka et al., Cell 43, 153-163 (1985); B.J. Classon et al., Int. Immunol. 4, 2, 215-225 (1992)). In the amino acid sequence set forth in SEQ ID No. 7 the hinge region (L γ t-2) extends from the amino acid at position 245 to the amino acid at position 304.

Additionally to the antigen binding domain and the hinge region the chimeric protein of the invention comprises a functional ζ domain contributing the transmembrane and the signalling domain of the chimeric protein. A functional ζ domain essentially comprises the transmembrane and the cytoplasmic domain of the ζ chain. The ζ domain mediated activation of the TCR by interaction of the antigen binding domain of the chimeric protein of the invention with a specific antigen triggers several signalling pathways, e.g. the ones mentioned above. According to the invention the ζ chain is of mammalian, particularly murine or human origin. Within the TCR ζ exists as a $\zeta\zeta$ disulphide homodimer. A functional ζ domain is a protein which upon expression in T cell hybridomas deficient in endogenous ζ expression is capable of restoring in said hybridomas a functionally active TCR, e.g. in such a way that antigen-induced interleukin-2 secretion and growth stimulation are regained (S. Frank et al., Science 249, 174-177 (1990)). Examples of a functional ζ domain include molecules comprising amino acids 28 to 164 of the murine (A.M. Weissman, Science 239, 1018-1021 (1988)) and amino acids 28 to 163 of the human ζ chain (numbering according to A.M. Weissmann et al., Proc. Natl. Acad. Sci. USA 85, 9709-9713 (1988), Fig. 2, which is incorporated herein by reference). It is envisaged that a ζ protein as used for the purpose of the present invention is intended to include variants with the provision that these variants are functional. Preferred are variants of mammalian, particularly murine and human origin.

For example, a variant is a naturally occurring variant of the ζ molecule as found within a particular species. Such a variant may be encoded by a related gene of the same gene family or an allelic variant of a particular gene. The term "variant" also embraces a modified ζ molecule producible from a DNA which has been subjected to in vitro mutagenesis, with the provision that the protein encoded by the DNA has the functional activity of the authentic ζ molecule. Such modifications may consist in an addition, exchange and/or deletion of one or more amino acids, the latter resulting in shortened variants.

A preferred chimeric protein of the invention comprises a protein having the amino acid sequence depicted in SEQ ID No. 7.

Moreover, the invention relates to a polyclonal and monoclonal antibody specifically binding to a protein of the invention. Such an antibody is prepared according to conventional methods well known in the art.

The chimeric protein of the invention may be prepared by a process that is known per se, characterized in that suitable host cells as defined further below producing a protein of the invention, are multiplied in vitro or in vivo, and, if desired, the protein is isolated.

Preferably, a protein of the invention is produced by a process comprising culturing suitable transduced CTL under conditions which allow the expression of the DNA construct encoding the protein and, optionally, performing an assay detecting the functionality of the protein. The invention further concerns a method for the manufacture of a chimeric protein of the invention comprising culturing a suitable host cell, particularly a CTL, which has been transduced with a vector comprising an expression cassette comprising a promoter and a DNA coding for said protein which DNA is controlled by said promoter under conditions which allow the expression of said DNA. A preferred chimeric protein of the invention is constructed to include a scFv, a hinge region and a functional ζ molecule. The process for producing the chimeric protein of the invention should yield the protein in an amount sufficient to enable the transduced host cell to lyse a target cell.

Suitable host cells include e.g. primary cytotoxic T lymphocytes (CD 8⁺), CD 4⁺ T helper cells and natural killer cells (NK). Preferred are mammalian cells, especially CTL of mammalian , particularly human origin.

As used hereinbefore or hereinafter, in vitro means ex vivo, thus including cell culture conditions.

For example, multiplication of mammalian cells in vitro is carried out in suitable culture media, which are customary standard culture media, such as Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. fetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder cells.

The invention also concerns a recombinant DNA or DNA construct suitable for manipulating the recognition specificity of T-lymphocytes. More specifically, the present invention provides a DNA construct capable of directing the synthesis of a chimeric protein comprising a recognition function, a hinge region and the ζ -chain as a signalling component of the TCR. In particular, the invention provides a DNA construct encoding a chimeric protein comprising an antigen binding domain, a hinge region and a ζ domain, particularly a DNA construct comprising at least one polynucleotide coding for a protein part designated as preferred hereinbefore or hereinbelow. In a preferred arrangement the antigen binding domain is conceived as the first part, the hinge region as the second part and the ζ chain as the third part.

By definition the DNAs of the invention include coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and DNA complementary thereto, or these complementary (single stranded) DNAs themselves.

Advantageously, the DNA construct of the invention comprises a fourth part which is located upstream of the first part (the antigen binding domain) and which encodes a leader peptide. Preferably, the fourth part of the DNA construct of the invention encodes a leader peptide of an immunoglobulin (Ig) gene, e.g. an Ig heavy chain leader peptide. The Ig heavy chain leader peptide promotes targeting of nascent polypeptides to the lumen of the endoplasmic reticulum; it is subsequently cleaved off and the protein is sorted through the Golgi and the membrane to its transmembrane location. Particularly preferred is a leader peptide having the sequence: Met-Ala-Trp-Val-Trp-Thr-Leu-Leu-Phe-Leu-Met-Ala-Ala-Ala-Lys-Val-Pro-Lys.

Preferred is a DNA comprising a DNA encoding the protein with the amino acid sequence depicted in SEQ ID No.7, e.g. a DNA having the nucleotide sequence depicted in SEQ ID No. 5. The DNA sequence set forth in SEQ ID No. 7 has the following features:
description of the sequence: 5'-EcoRI-IgH chain leader D6/12-scFv(FRP5):Lyt-2 hinge:-CD3 zeta(transmembrane (TM) and cytoplasmic (Cyt))-EcoRI-3'

5'EcoRI site:	position 1
3'RcoRI site:	position 1474
ATG initiation	position 40
TAA stop	position 1423
IGH chain leader	position 40-93

scFv(FRP5)	position 94-819
lyt-2 hinge insert	XbaI (position 819)-
	XbaI (position 1005)
zeta insert	XbaI (position 1005)-
	EcoRI (position 1474)

The present state of the art is such that a person with ordinary skill in the art will be able to synthesize a DNA molecule of the invention given the written information provided herein. A suitable method for obtaining a DNA construct of the invention involves methods well-known in the art comprising e.g. synthesis of a number of oligonucleotides, amplification of specific gene sequences, e.g. using PCR (polymerase chain reaction) technology, their splicing to give the desired DNA sequence and/or use of DNA restriction enzymes and ligases. A DNA of the invention may be synthesized by combining chemical with recombinant methods.

The invention further concerns a vector, such as a retroviral vector, comprising a DNA construct of the invention.

Additionally, the present invention provides a genetically engineered transduced CTL which is capable of destroying a targeted tumor cell in an MHC-independent and MHC-unrestricted manner. According to the present invention, the CTL produces the above-identified chimeric protein of the invention. The CTL is transduced with a DNA of the invention and thus is capable of expressing said DNA and of producing the protein encoded by said DNA. Destruction of the targeted tumor cell requires that the protein thus produced is functional, i.e. the antigen binding domain of said protein must be capable of recognizing and binding to the targeted tumor cell and the ζ domain must be capable of triggering the desired signal within the CTL. The CTL of the invention is cultured under conditions enabling (favoring) the expression of the introduced DNA and, if desired, assayed for the production thereof. Prolonged and elevated expression of said DNA is preferred.

Furthermore, the present invention provides a process for endowing a CTL with a defined, MHC-independent and MHC-unrestricted tumor cell specificity by introducing into said T-lymphocyte a DNA construct comprising a recognition function, a spacer domain and the ζ -chain as a signalling component of the TCR. The DNA construct may be introduced into the CTL by DNA-transfer methods apparent to those skilled in the art, e.g. by means

of a vector system, such as a viral or non-viral vector system. Suitable viral vectors include retroviral, adenoviral and adeno-associated viral vectors. The process is applicable to both in vivo and in vitro situations. In vitro application is preferred.

The T-lymphocyte is cultured under conventional conditions allowing the expression of said DNA construct and assayed for the production thereof. Prolonged and elevated expression of said DNA is preferred. Advantageously, CTL are cultured in the presence of IL-2. Transduced CTL of the invention may be selected for a suitable marker. For example, the transduced CTL may be selected for the cotransduced neo resistance marker if the DNA construct of the invention is transferred via a retroviral vector.

Moreover, the invention relates to a composition of matter comprising the transduced CTL of the invention. Such a composition comprises e.g. transduced CTL producing a protein of the invention together or in admixture with an acceptable, e.g. a pharmaceutically acceptable, carrier. Such a carrier may be a solid or liquid carrier. The composition may be used ex vivo, e.g. in order to kill preselected target cells in a composition (for example body liquid or tissue) removed from a patient's body. After the target cells have been killed (which should be checked) the composition is re-introduced into the patient's body. Thus the composition of the invention may be used for the treatment or adjuvant treatment of tumors.

Additionally, the present invention provides a process for lysing selected tumor cells comprising contacting said tumor cells to CTL producing the chimeric protein of the invention. In the process which is applicable to both in vitro and in vivo situations the tumor cell is targeted by the antigen binding domain which is part of the chimeric protein of the invention.

It is preferred to use the host's own CTL, particularly if the exposure and interaction is to occur in vivo, but, if appropriate, the CTL may also be derived from other sources. Other sources are e.g. tissue culture or another mammal of the same or different species.

CTL are found throughout the body of the mammal: in tissues, the lymphatic system and in the blood. Suitable CTL are selected and removed from the mammal. For example, CTL are selected as CD8⁺ peripheral lymphocytes cultured in vitro in the presence of IL-2. Alternatively, unselected peripheral lymphocytes are used for gene transduction. If desired, the host may be treated such as to increase the number of stimulated CTL.

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The invention further concerns a method of treating cancer comprising the use of the genetically engineered CTL of the invention. The method comprises exposing selected tumor cells to CTL producing the chimeric protein of the invention. An in vitro (meaning ex vivo) application of this method for promotion of CTL-mediated lysis may be in the selective treatment of tumor cells removed from a mammal, particularly a human, in the need of cancer treatment. An example would be to use the CTL of the invention to eliminate tumor cells from bone marrow removed from a patient, e.g. a patient undergoing radiation treatment prior, to re-introducing the bone marrow. As a consequence of the interaction of the tumor cells and the CTL of the invention the tumor cells are lysed. If the method of treating cancer is performed in vivo it may further comprise re-introducing the transduced CTL of the invention into the body of the mammal, particularly the human, to be treated. It is also envisaged that CTL expressing a DNA of the invention are produced by in vivo transduction of the DNA, e.g. in a mammal in need of cancer treatment.

The invention further concerns the CTL of the invention or a composition comprising said CTL for use in a method of treating cancer.

The invention particularly concerns the specific embodiments (e.g. protein, DNA, CTL and methods for the preparation thereof) described in the Examples. The following examples illustrate the invention but do not limit it to any extent.

Abbreviations: FCS: fetal calf serum; LDH: lactate dehydrogenase; mAb: monoclonal antibody; MoMLV: Moloney murine leukemia virus; MoMLV-LTR: Moloney murine leukemia virus-long terminal repeat, scFv: single chain antibody; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis.

Materials and Methods

Cell Lines and Culture Conditions

Clone 96 (CI96) is a H-2K^d-restricted cytotoxic T cell line derived from C57BL/6 mice (K. Eichmann et al., J. Immunol. 147, 2075-2081 (1991)). CI96 and infectants are maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS (Boehringer), 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine and 3% conditioned supernatant obtained from X63Ag8-653 plasmacytoma cells transfected with the murine IL-2 cDNA (H. Karasuyama and F. Melchers, Eur. J. Immunol. 18, 97-104 (1988)). The human leukemic T cell line Jurkat, the retroviral

packaging cell lines Ω E (J.P. Morgenstern and H. Land, Nuc. Acids Res. 18, 3587-3596 (1990)) and P317 (A.D. Miller and G.J. Rosman, Biotechniques 7, 980-990 (1989)) and infectants and the murine fibroblast cell line transfected with the activated human erbB-2 receptor, NIH3T3#3.7 are cultured in DMEM supplemented with 10% FCS. HC11R1#11 is a mouse mammary epithelial cell line transfected with the human erbB-2 proto-oncogene (N.E. Hynes et al., Mol. Cell. Biol. 10, 4027-4034 (1990)) which is grown in RPMI 1640 (Gibco) supplemented with 10% FCS, 10 ng/ml epidermal growth factor and 5 μ g/ml insulin.

Example 1: Construction of the scFv(FRP5):hinge:zeta(ζ) cDNA

A DNA consisting of a recognition function, a spacer domain and the ζ -chain as a signalling component of the TCR/CD3 receptor complex is constructed. The recognition function is contributed by a scFv domain. This domain is derived from the monoclonal antibody FRP5 (European patent application EP-A-502 812). FRP5 is specific for the extracellular domain of the erbB-2 receptor. The scFv (FRP5) comprises the variable domains of the heavy and light chains (V_H and V_L) of the monoclonal antibody (mAb) joined by a 15 amino acid linker sequence (SEQ ID NO:2). This scFv domain is able to recognize the extracellular domain of the erbB-2 receptor (W. Wels et al., Biotechnology 10, 1128-1132 (1992); W. Wels et al., Cancer Res. 15, 6310-6317 (1992)). A leader sequence from an immunoglobulin heavy chain is added to the N-terminus of the scFv domain. The scFv(FRP5) cDNA is ligated to a short linker sequence encoding 59 amino acids from the immunoglobulin-like hinge region of the CD8 α gene (R. Zomoyka et al., Cell 43, 153-163 (1985)). The transmembrane and signalling domain are contributed by the ζ -chain of the TCR. This chain is responsible for the signal transduction following TCR activation.

The cDNA encoding the single chain antibody FRP5 specific for the extracellular domain of the erbB-2 molecule (SEQ ID NO:1) is subcloned into a plasmid containing an immunoglobulin heavy chain leader (L_{IgH}). Both, the ζ cDNA and the CD8 α hinge cDNA are derived from total RNA of the cytotoxic T cell line Cl96 using a combination of reverse transcription and the polymerase chain reaction (RT-PCR). MoMLV reverse transcriptase is used for first strand cDNA synthesis. The reactions are primed with the 3' ζ -specific oligonucleotide 5813 (SEQ ID NO:6) or the 3' CD8 α -specific oligonucleotide 8764 (SEQ ID NO:7), respectively. These cDNAs are used as PCR templates with the ζ primer pair 5812/5813 (SEQ ID NOs. 8 and 6) introducing a 5' XbaI site and a 3' HindIII/BglII site and the CD8 α hinge primer pair 8763/8764 (SEQ ID NOs. 10 and 8)

introducing a XbaI site at both ends. The L_{IgH}-scFv(FRP5) DNA is ligated to the ζ cDNA starting from amino acid residue 28 (numbering according to A.M. Weissman et al., Science 239, 1018-1021 (1988)) using the XbaI site for the fusion. The CD8α hinge cDNA encoding amino acid residues 105 to 164 (numbering according to R. Zomoyka et al., Cell 43, 153-163 (1985)) is subsequently inserted into the XbaI site and checked for right orientation. The resulting scFv:hinge:ζ cDNA construct (SEQ ID No. 7) is confirmed by complete DNA sequencing and eventually subcloned into the unique EcoRI site of the pLXSN retroviral vector (A.D. Miller and G.J. Rosman, supra) resulting in the pL(scFv(FRP5):hinge:ζ)SN construct. The expression of the DNA is controlled by the 5' MoMLV-LTR. The plasmid also bears a selectable marker for neomycin resistance driven by the SV40 early promoter (SN).

Cloning of pL(scFv:D3/D4:ζ)SN

While the first molecular design includes the relatively short and flexible immunoglobulin hinge-like region of the murine Lyt-2 or CD8α molecule as tether, the second design encompasses the two membrane-proximal immunoglobulin-like domains designated as D3 and D4 of the murine L3T4 or CD4 molecule (S.J. Clark et al., Proc. Natl. Acad. Sci. USA 84, 1649-1653 (1987)) as a longer and more rigid spacer. D3/D4 encoding cDNA is obtained by PCR using pcd-L3T4 4.25 plasmid DNA (D.R. Littman and S.N. Gettner, Nature 325, 453-455 (1987)). The specific primer pair #8761/#8762 amplifies the coding sequences for amino acid residues 184-370 of the CD4 molecule (P.J. Maddon et al., Proc. Natl. Acad. Sci. USA 84, 9155-9159 (1987)) introducing XbaI restriction sites at both ends of the cDNA. The product is subcloned into the XbaI site of the pL(FZ)SN vector. After checking for the correct orientation of the insert, sequence identity of the resulting construct is confirmed by DNA sequencing. The structure of the pL(F4Z)SN is shown in Figure 1.

Primers for the amplification of the L3T4/CD4D3/D4 cDNA:

'Upstream' -5' Lyt-2/CD8-specific oligonucleotide #8761¹⁾:

#8761: 5'-AGCTTCTAGAGTTTCAGAGCACAGCTCTCACGGCC-3'

'Downstream' -3' Lyt-2/CD8-specific oligonucleotide #8762¹⁾:

#8762: 5'-TCGATCTAGAGTCTGGTTCACCCCTCTGG-3'

¹⁾ XbaI restriction sites are underlined.

Example 2: Expression of the scFv(FRP5):hinge:ζ DNA after retroviral gene transfer

The pLXSN vector system is capable of directing the efficient synthesis of the scFv(FRP5):hinge:ζ DNA after transduction into cytotoxic T cells and allows for G418 selection of infected cells. An established murine CTL line, CI96, is infected with the pL(scFv(FRP5):hinge:ζ)SN construct of Example 1.

The ecotropic packaging cell line ΩE is transfected by calcium-phosphate precipitation with pL(scFv(FRP5):hinge:ζ)SN plasmid DNA. Transfected cells are stably selected in the presence of the neomycin analogue G418 (Genitacin, 1 mg/ml, Gibco). Viral supernatants are harvested after 48 hours from pools of G418 resistant helper cells and used to infect the amphotropic packaging cell line PA317 in the presence of 8 mg/ml polybrene. Clonal, high titer producer lines are derived by selection in 1.0 mg/ml G418 containing medium. Supernatants of these producer lines are used to infect CI96 cells. Clones of infected cells selected for high expression of the scFv(FRP5):hinge:ζ DNA are derived and assayed for the production of chimeric cell surface proteins (Example 3). Clone CFYZ.1 is derived by growth in 1.0 mg/ml G418. Jurkat cells are infected using the same procedure, the clone JFYZ.4 is derived by growth in 2.0 mg/ml G418.

Example 3: Biochemical characterization of cell surface proteins

a) SDS-PAGE analysis of the chimeric scF(FRP5):hinge:ζ proteins produced by transduced CTL

Selected clones of Example 2 are cell surface biotinylated, lysed and immunoprecipitated with an anti-ζ mAb. For surface biotinylation, 3×10^7 viable cells are washed three times in biwa buffer (PBS, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and resuspended in 1.5 ml Sulfo-NHS Biotin in biwa buffer (Pierce, 0.5 mg/ml). After incubating 15 min at 4°C, the reaction is quenched by addition of 25 mM L-lysine in biwa buffer. The cells are washed three times in quenching buffer (25 mM L-lysine in biwa buffer) and lysed in 1% NP-40, 150 mM NaCl, 50 mM Tris/HCL pH 8.0, 5 mM EDTA, 1 mM PMSF containing buffer supplemented with a protease inhibitor cocktail. Postnuclear lysates are precleared overnight with Protein A Sepharose (Pharmacia). Immunoprecipitation is performed by addition of 3 mg of the ζ-specific mAb H146-968 which recognizes human and mouse COOH terminus of the ζ chain, incubated for 3 hours followed by 1 hour incubation with Protein A Sepharose. The precipitate is washed four times in NET-TON (650 mM NaCl, 5 mM EDTA, 50 mM Tris/HCL, 0.5% Triton X-100, 1 mg/ml ovalbumin). For deglycosylation, precipitates are denatured in 5% SDS with or without 10%

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2-mercaptoethanol at 100°C and incubated with 2.000 U PNGase F (Biolabs) for 1 hour at 37°C. Samples are boiled in either non-reducing or reducing Laemmli-sample buffer and electrophoresed through 5-20% SDS-PAGE gradient gels. The proteins are transferred to a PVDF membrane (Millipore) and blocked in PBS-T (PBS, 0.4% Tween-20) containing 5% skim milk powder (Fluka). The membrane is incubated for one hour with PBS-T containing horseradish peroxidase-streptavidin (HRP-Strep, Southern Biotechnology, 1:5.000). After washing the membrane four times for 7 min in PBS-T, the blot is developed using the ECL-chemoluminescence reagent (Amersham). The SDS-PAGE analysis of the immunoprecipitates under reducing conditions reveals a series of bands with an apparent molecular weight of about 48-65 kDa from lysates of infected cells (clone CFYZ.1), but not in lysates of the parental cells (Cl96 cells). The 48 kDa band corresponds to the scFv(FRP5):hinge:ζ protein with a calculated molecular weight of 48.7 kDa. The amino acid sequence of said protein is depicted in SEQ ID NO:5. In said sequence listing the recognition part derived from mAb FRP5 extends from the amino acid at position 6 (Gln) to the amino acid at position 240 (Ile), the hinge region derived from CD8α extends from the amino acid at position 245 (Ile) to the amino acid at position 304 (Phe) and the ζ chain extends from the amino acid at position 307 (Asp) to the amino acid at position 443 (Arg). The higher molecular weight species arise as a consequence of complex glycosylation of the scFv and the hinge region. Deglycosylation with the endoglycosidase PNGase F results in a simplified protein pattern and the reduction of the apparent molecular weight to about 47 kDa. The endogenous ζ-chain is detected as a 16 kDa band (16.3 kDa predicted) in uninfected and infected cells. When the SDS-PAGE analysis is carried out under non-reducing conditions, both disulfide-linked scFv:hinge:ζ homodimers with an apparent molecular weight of about 96 kDa as well as heterodimers of scFv(FRP5):hinge:ζ molecules with the endogenous ζ with an apparent molecular weight of about 64 kDa are observed. PNGase F treatment slightly reduced the molecular weights of these two bands. The detected 32 kDa band corresponds to endogenous ζ-ζ homodimers of the CTL.

b) Flow cytometric analysis of scFv(FRP5):hinge:ζ protein producing T cells

Cell surface expression and erbB-2 receptor binding ability of the scFv(FRP5):hinge:ζ protein in transduced Cl96 CTL and in transduced Jurkat cells are confirmed by flow cytometry.

Single-cell suspensions of 5×10^5 viable cells (Jurkat cells, JFYZ.4 cells, Cl96 cells, CFYZ.1 cells) are stained with the purified extracellular domain of the erbB-2 protein (erbB-2^{ecd}, expressed in Sf9 insect cells using a baculovirus expression vector; Disis et

al., Cancer Res. 54, 16-20 (1994)) for 1 hour followed by the FITC-conjugated anti-erbB-2 monoclonal antibody FSP77 (European patent application EP-A-502 812) for 45 min at 4°C in 100 µl PBS containing 1% BSA and 0.1% sodium azide. FSP77 also is specific for the extracellular domain of the erbB-2 receptor, but recognizes a different epitope from mAb FRP5 (I.M. Harwerth et al., J. Biol. Chem. 21, 15160-15167 (1992)). Ten thousand forward scatter/side scatter gated viable cells are acquired and analysed with a flow cytometer revealing binding of the purified, soluble extracellular domain of the erbB-2 receptor to the transduced T cells JFYZ.4 and CFYZ.1 but not to non-infected Jurkat or CI96 cells.

The hinge region provides flexibility and accessibility to the scFv moiety and is a necessary prerequisite for the binding of the extracellular domain of the erbB-2 receptor to the scFv domain. Insertion of the CD4 D3D4 also allows binding. A construct in which a direct fusion, without a hinge region or spacer, of the scFv domain to the ζ-chain is tested, results in a surface receptor which cannot bind to the erbB-2 protein.

Example 4: Signal transduction of the scFv(FRP5):hinge:ζ fusion protein

The intracellular calcium (Ca^{2+}) concentration of T cells loaded with a suitable calcium-chelating fluorescent dye is measured after incubation with the soluble erbB-2 receptor. For this purpose cultured JFYZ.4 infectants and Jurkat cells are suspended at $1 \times 10^7/\text{ml}$ in RPMI 1640 supplemented with 2% FCS and 5 mM Indo-1/AM (Calbiochem) (M. Lopez et al., Cytometry 10, 165-173 (1989)) and rotated for 45 min at 37°C. After washing twice, 3×10^5 cells are incubated on ice with 2 mg purified erbB-2^{ecd}. Triggering is performed at 37°C by simultaneous administration of 5 mg anti-erbB-2 mAb FSP77 followed by crosslinking with a goat anti-mouse Ig antiserum (GαM Ig, Southern Biotechnology). As a control, cells are triggered by addition of anti-human CD3ε mAb (Serva) and GαM Ig. Calcium flux is monitored for 15 min on a flow cytometer by measuring emission at 405 and 525 nm.

Crosslinking results in a rapid increase of intracellular calcium in JFCZ.4 cells but not in parental Jurkat cells comparable to that obtained by crosslinking the CD3 complex with an anti-CD3ε mAb in non-infected cells. This indicates that intracellular signalling is triggered upon crosslinking of the scFv(FRP5):hinge:ζ protein via an extracellular ligand domain and that the scFv(FRP5):hinge:ζ protein is functionally active.

Example 5: In vitro cytotoxicity assay

The cytolytic activity of infected CI96 (CFYZ.1) cells is determined in vitro.

Oncogenically transformed mouse NIH/3T3 fibroblasts and HC11 epithelial cells expressing the human erbB-2 receptor (N.E. Hynes et al., supra)) are employed as target cells. The release of LDH from these cells is used as a measure of cell lysis (T. Decker and M.L. Lohmann-Matthes, J. Immunol. Methods 115, 61-69 (1988)).

The cytotoxicity assay is performed in phenol red free medium supplied with 4% conditioned supernatant containing recombinant murine IL-2 (rmIL-2, see above). A constant number of target cells (7.500/well) is added to a serial 2-fold dilution of effectors (CFYZ.1 cells) followed by an eight hour incubation at 37°C and 5% CO₂. All dilutions are performed in triplicates. The LDH content of a 50 µl aliquot of the supernatant is assayed using the CytoTox 96 assay (Promega) (T. Decker and M.L. Lohmann-Matthes, supra). The LDH activity measured after lysis of target cells with 0.4% Triton X-100 is considered as 100%. The measured experimental values are corrected for the spontaneous release of LDH from effector and target cells. Infected CI96 cells expressing the scFv(FRP5):hinge:ζ construct efficiently lyse erbB-2 expressing NIH/3T3 cells or HC11 cells at effector to target ratios between 1 and 10. Lysis of the epithelial and fibroblast target cells transfected with the human erbB-2 receptor occurs in a non MHC-restricted manner indistinguishable from normal antigen-specific cellular cytotoxicity. In contrast, no cell lysis is observed when the parental CI96 cells are used as effectors. The mAb FRP5 and the derived scFv domain are specific for the human erbB-2 molecule and do not recognize the mouse homologue which is expressed at low levels on both cell lines. For this reason, no cell lysis is observed when untransfected NIH/3T3 cells or HC11 cells are incubated with the scFv(FRP5):hinge:ζ construct expressing T cells.

Example 6: In vivo anti-tumor activity

Two experimental schedules are used to assess the anti-tumor activity of the transduced CTL (infected CI96 cells) in vivo. In the first schedule, 5×10^5 NIH3T3#3.7 tumor cells are mixed with 5×10^6 CFYZ.1 cells or parental CI96 cells (effector to target ratio of 1:10) in 0.1 ml culture medium and immediately injected subcutaneously (s.c.) into the right flank of Balb/c nude mice (H.J. Winn, J. Immunol. 86, 228-234 (1961)). The growth of the tumors is followed by caliper measurements. NIH3T3#3.7 tumor cells alone are injected as a control. Each group consists of five animals. In the second schedule, Balb/c nude mice are inoculated s.c. into the right flank with 4×10^5 NIH3T3#3.7 tumor cells. On day 4 and 5, when tumors are palpable, parental CI96 cells and CFYZ.1 cells are injected intravenously into the tail vein (1×10^7 cells in 0.2 ml culture medium). 500 U of rhIL-2 (Hoffmann-La Roche) in 0.2 ml PBS are administered intraperitoneally on days 4, 5 and 6. The growth of the tumors is followed by caliper measurements. NIH3T3#3.7 cells

without Il-2 and with Il-2 are injected as controls. Each group consists of five animals. NIH/3T3 cells transformed with the human erbB-2 oncogene lead to the rapid formation of tumors after subcutaneous injection into athymic Balb/c nude mice. The simultaneous administration of CFYZ.1 infectants and tumor cells completely suppresses tumor formation for up to 7 days. Administration of the uninfected parental C196 cells, however has no effect on tumor cell growth. A similar result is obtained when nude mice are inoculated first with NIH/3T3-erbB-2 tumor cells and subsequently treated with CFYZ.1 cells in combination with exogenous Il-2. The administration of the transduced CTL strongly retards the growth of the tumor cells over a course of seven days thus showing a systemic in vivo effect. The cells have the capability to home the tumor, to be activated and display their cytolytic activity when administered at a different site.

These results show that the specificity and thus the cytolytic effector machinery of the transduced CTL can be efficiently redirected towards a predefined surface antigen, the erbB-2 receptor, which plays an important role in the etiology of many human adenocarcinomas including breast, ovarian, gastric and colon cancer. Therefore the principle of targeted T cell action is conceived as a useful therapy approach and generally applicable for the elimination of tumor cells which express a surface antigen at higher levels than normal cells. The design permits the generation of CTL with many desired specificities by exchanging the scFv moiety and replacing it with any existing antigen recognition function derived from a specific monoclonal antibody. The use of efficient transfer systems, e.g. retroviral vectors, allows the transfer of scFv:hinge:ζ DNAs into cell types which are not easily transfectable.

Deposition data:

Hybridoma cell lines producing antibodies FRP5, FSP16, FSP77 and FWP51 have been deposited with the European Collection of Animal Cell Cultures (ECACC, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, UK) on November 21, 1990 under accession numbers 90112115, 90112116, 90112117 and 90112118, respectively.

Brief Description of the Figure:

Figure 1: Structure of the pL(F4Z)SN retroviral vector. A cDNA encoding amino acid residues number 184 - 370 of the CD 4 immunoglobulin like D3 and D4 domains is derived by PCR and subcloned into the XbaI site of the PL(FX)SN vector. Amino acid sequences of the fusion boundaries are shown in the single letter code.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: CIBA-GEIGY AG
- (B) STREET: Klybeckstr. 141
- (C) CITY: Basel
- (E) COUNTRY: SCHWEIZ
- (F) POSTAL CODE (ZIP): 4002
- (G) TELEPHONE: +41 61 69 11 11
- (H) TELEFAX: + 41 61 696 79 76
- (I) TELEX: 962 991

(ii) TITLE OF INVENTION: Bifunctional Protein, Preparation and Use

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 748 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 9..728
- (D) OTHER INFORMATION: /product= "scFv(FRP)5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAGCTTCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG AAG AAG CCT
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro
1 5 10

005T50"4296560

GGA	GAG	ACA	GTC	AAG	ATC	TCC	TGC	AAG	GCC	TCT	GGG	TAT	CCT	TTC	ACA	98
Gly	Glu	Thr	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Pro	Phe	Thr	
15					20					25					30	
AAC	TAT	GGA	ATG	AAC	TGG	GTG	AAG	CAG	GCT	CCA	GGA	CAG	GGT	TTA	AAG	146
Asn	Tyr	Gly	Met	Asn	Trp	Val	Lys	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Lys	
				35					40					45		
TGG	ATG	GGC	TGG	ATT	AAC	ACT	TCC	ACT	GGA	GAG	TCA	ACA	TTT	GCT	GAT	194
Trp	Met	Gly	Trp	Ile	Asn	Thr	Ser	Thr	Gly	Glu	Ser	Thr	Phe	Ala	Asp	
			50					55					60			
GAC	TTC	AAG	GGA	CGG	TTT	GAC	TTC	TCT	TTG	GAA	ACC	TCT	GCC	AAC	ACT	242
Asp	Phe	Lys	Gly	Arg	Phe	Asp	Phe	Ser	Leu	Glu	Thr	Ser	Ala	Asn	Thr	
		65					70					75				
GCC	TAT	TTG	CAG	ATC	AAC	AAC	CTC	AAA	AGT	GAA	GAC	ATG	GCT	ACA	TAT	290
Ala	Tyr	Leu	Gln	Ile	Asn	Asn	Leu	Lys	Ser	Glu	Asp	Met	Ala	Thr	Tyr	
	80					85					90					
TTC	TGT	GCA	AGA	TGG	GAG	GTT	TAC	CAC	GGC	TAC	GTT	CCT	TAC	TGG	GGC	338
Phe	Cys	Ala	Arg	Trp	Glu	Val	Tyr	His	Gly	Tyr	Val	Pro	Tyr	Trp	Gly	
95					100					105					110	
CAA	GGG	ACC	ACG	GTC	ACC	GTT	TCC	TCT	GGC	GGT	GGC	GGT	TCT	GGT	GGC	386
Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	
				115					120					125		
GGT	GGC	TCC	GGC	GGT	GGC	GGT	TCT	GAC	ATC	CAG	CTG	ACC	CAG	TCT	CAC	434
Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Asp	Ile	Gln	Leu	Thr	Gln	Ser	His	
			130					135					140			
AAA	TTC	CTG	TCC	ACT	TCA	GTA	GGA	GAC	AGG	GTC	AGC	ATC	ACC	TGC	AAG	482
Lys	Phe	Leu	Ser	Thr	Ser	Val	Gly	Asp	Arg	Val	Ser	Ile	Thr	Cys	Lys	
		145					150					155				
GCC	AGT	CAG	GAT	GTG	TAT	AAT	GCT	GTT	GCC	TGG	TAT	CAA	CAG	AAA	CCA	530
Ala	Ser	Gln	Asp	Val	Tyr	Asn	Ala	Val	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	
	160					165					170					
GGA	CAA	TCT	CCT	AAA	CTT	CTG	ATT	TAC	TCG	GCA	TCC	TCC	CGG	TAC	ACT	578
Gly	Gln	Ser	Pro	Lys	Leu	Leu	Ile	Tyr	Ser	Ala	Ser	Ser	Arg	Tyr	Thr	
175					180					185					190	
GGA	GTC	CCT	TCT	CGC	TTC	ACT	GGC	AGT	GGC	TCT	GGG	CCG	GAT	TTC	ACT	626
Gly	Val	Pro	Ser	Arg	Phe	Thr	Gly	Ser	Gly	Ser	Gly	Pro	Asp	Phe	Thr	
				195					200					205		
TTC	ACC	ATC	AGC	AGT	GTG	CAG	GCT	GAA	GAC	CTG	GCA	GTT	TAT	TTC	TGT	674
Phe	Thr	Ile	Ser	Ser	Val	Gln	Ala	Glu	Asp	Leu	Ala	Val	Tyr	Phe	Cys	
			210					215					220			

CAG CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG ACA AAA TTG 722
Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu
225 230 235

GAG ATC TAGCTGATCA AAGCTCTAGA 748
Glu Ile
240

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 240 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
1 5 10 15
Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn Tyr
20 25 30
Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp Met
35 40 45
Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe
50 55 60
Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr
65 70 75 80
Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys
85 90 95
Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln Gly
100 105 110
Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
115 120 125
Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser His Lys Phe
130 135 140
Leu Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser
145 150 155 160
Gln Asp Val Tyr Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln
165 170 175

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Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val
180 185 190

Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe Thr
195 200 205

Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln
210 215 220

His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile
225 230 235 240

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 748 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 9..728
- (D) OTHER INFORMATION: /product= "scFv (FWP51)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAGCTTCT CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG CTG GTG AGG CCT	50
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro	
1 5 10	
GGG ACT TCA GTG AAG CTG TCC TGC AAG GCT TCT GAT TAC ACC TTC ACC	98
Gly Thr Ser Val Lys Leu Ser Cys Lys Ala Ser Asp Tyr Thr Phe Thr	
15 20 25 30	
AGC TAC TGG ATG AAC TGG GTG AAG CAG AGG CCT GGA CAA GGC CTT GAA	146
Ser Tyr Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu	
35 40 45	
TGG ATT GGT ATG ATT GAT CCT TCA GAC AGT GAA ACT CAA TAC AAT CAA	194
Trp Ile Gly Met Ile Asp Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln	
50 55 60	
ATG TTC AAG GAC AAG GCC GCA TTG ACT GTA GAC AAG TCC TCC AAT ACA	242
Met Phe Lys Asp Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr	
65 70 75	

006T90" 4496560

006790"4296560

GCC TAC ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT	290
Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr	
80 85 90	
TAC TGT GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT GTC TGG	338
Tyr Cys Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp	
95 100 105 110	
GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT GGT	386
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly	
115 120 125	
GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG CTG ACC CAG TCT	434
Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser	
130 135 140	
CCA TCC TCA CTG TCT GCA TCT CTG GGA GGC GAA GTC ACC ATC ACT TGC	482
Pro Ser Ser Leu Ser Ala Ser Leu Gly Gly Glu Val Thr Ile Thr Cys	
145 150 155	
AAG GCA AGC CAA GAC ATT AAG AAG TAT ATA GCT TGG TAC CAA CAC AAG	530
Lys Ala Ser Gln Asp Ile Lys Lys Tyr Ile Ala Trp Tyr Gln His Lys	
160 165 170	
CCT GGA AAA AGT CCT CGG CTA CTC ATA CAC TAC ACA TCT GTA TTA CAG	578
Pro Gly Lys Ser Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu Gln	
175 180 185 190	
CCA GGC ATC CCA TCC AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT	626
Pro Gly Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr	
195 200 205	
TCC TTC AGC ATC CAC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT	674
Ser Phe Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr	
210 215 220	
TGT CTA CAT TAT GAT TAT CTG TAC ACG TTC GGA GGG GGC ACC AAG CTG	722
Cys Leu His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu	
225 230 235	
GAG ATC TAGCTGATCA AAGCTCTAGA	748
Glu Ile	
240	

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 240 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Leu Ser Cys Lys Ala Ser Asp Tyr Thr Phe Thr Ser Tyr
20 25 30
Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Met Ile Asp Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe
50 55 60
Lys Asp Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr
65 70 75 80
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
85 90 95
Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp Gly Gln
100 105 110
Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
115 120 125
Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser
130 135 140
Ser Leu Ser Ala Ser Leu Gly Gly Glu Val Thr Ile Thr Cys Lys Ala
145 150 155 160
Ser Gln Asp Ile Lys Lys Tyr Ile Ala Trp Tyr Gln His Lys Pro Gly
165 170 175
Lys Ser Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro Gly
180 185 190
Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe
195 200 205
Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu
210 215 220
His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
225 230 235 240

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1479 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 40..1422
(D) OTHER INFORMATION: /product= "leader-scFv(FRP5):lyt-2
hinge:zeta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

006T90"4245550

GAATTCGGCA CGAGCTTAAG GCACCACTTC TTAGACATC ATG GCT TGG GTG TGG	54
Met Ala Trp Val Trp	
1 5	
ACC TTG CTA TTC CTG ATG GCA GCT GCC AAA GTG CCC AAG CAG ATC CAG	102
Thr Leu Leu Phe Leu Met Ala Ala Ala Lys Val Pro Lys Gln Ile Gln	
10 15 20	
TTG GTG CAG TCT GGA CCT GAG CTG AAG AAG CCT GGA GAG ACA GTC AAG	150
Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu Thr Val Lys	
25 30 35	
ATC TCC TGC AAG GCC TCT GGG TAT CCT TTC ACA AAC TAT GGA ATG AAC	198
Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn Tyr Gly Met Asn	
40 45 50	
TGG GTG AAG CAG GCT CCA GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT	246
Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp Met Gly Trp Ile	
55 60 65	
AAC ACC TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA CGG	294
Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly Arg	
70 75 80 85	
TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT TTG CAG ATC	342
Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu Gln Ile	
90 95 100	
AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC TGT GCA AGA TGG	390
Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys Ala Arg Trp	
105 110 115	
GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG GGC CAA GGG ACC ACG GTC	438
Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln Gly Thr Thr Val	
120 125 130	

006T30"4256560

ACC	GTT	TCC	TCT	GGC	GGT	GGC	GGT	TCT	GGT	GGC	GGT	GGC	TCC	GGC	GGT	486
Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	
135						140					145					
GGC	GGT	TCT	GAC	ATC	CAG	CTG	ACC	CAG	TCT	CAC	AAA	TTC	CTG	TCC	ACT	534
Gly	Gly	Ser	Asp	Ile	Gln	Leu	Thr	Gln	Ser	His	Lys	Phe	Leu	Ser	Thr	
150					155					160					165	
TCA	GTA	GGA	GAC	AGG	GTC	AGC	ATC	ACC	TGC	AAG	GCC	AGT	CAG	GAT	GTG	582
Ser	Val	Gly	Asp	Arg	Val	Ser	Ile	Thr	Cys	Lys	Ala	Ser	Gln	Asp	Val	
				170					175					180		
TAT	AAT	GCT	GTT	GCC	TGG	TAT	CAA	CAG	AAA	CCA	GGA	CAA	TCT	CCT	AAA	630
Tyr	Asn	Ala	Val	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ser	Pro	Lys	
			185					190					195			
CTT	CTG	ATT	TAC	TCG	GCA	TCC	TCC	CGG	TAC	ACT	GGA	GTC	CCT	TCT	CGC	678
Leu	Leu	Ile	Tyr	Ser	Ala	Ser	Ser	Arg	Tyr	Thr	Gly	Val	Pro	Ser	Arg	
		200					205					210				
TTC	ACT	GGC	AGT	GGC	TCT	GGG	CCG	GAT	TTC	ACT	TTC	ACC	ATC	AGC	AGT	726
Phe	Thr	Gly	Ser	Gly	Ser	Gly	Pro	Asp	Phe	Thr	Phe	Thr	Ile	Ser	Ser	
	215					220					225					
GTG	CAG	GCT	GAA	GAC	CTG	GCA	GTT	TAT	TTC	TGT	CAG	CAA	CAT	TTT	CGT	774
Val	Gln	Ala	Glu	Asp	Leu	Ala	Val	Tyr	Phe	Cys	Gln	Gln	His	Phe	Arg	
230					235					240					245	
ACT	CCA	TTC	ACG	TTC	GGC	TCG	GGG	ACA	AAA	TTG	GAG	ATC	AAA	GCT	CTA	822
Thr	Pro	Phe	Thr	Phe	Gly	Ser	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Ala	Leu	
				250					255					260		
GAG	ATC	AGC	AAC	TCG	GTG	ATG	TAC	TTC	AGT	TCT	GTC	GTG	CCA	GTC	CTT	870
Glu	Ile	Ser	Asn	Ser	Val	Met	Tyr	Phe	Ser	Ser	Val	Val	Pro	Val	Leu	
			265					270					275			
CAG	AAA	GTG	AAC	TCT	ACT	ACT	ACC	AAG	CCA	GTG	CTG	CGA	ACT	CCC	TCA	918
Gln	Lys	Val	Asn	Ser	Thr	Thr	Thr	Lys	Pro	Val	Leu	Arg	Thr	Pro	Ser	
		280					285					290				
CCT	GTG	CAC	CCT	ACC	GGG	ACA	TCT	CAG	CCC	CAG	AGA	CCA	GAA	GAT	TGT	966
Pro	Val	His	Pro	Thr	Gly	Thr	Ser	Gln	Pro	Gln	Arg	Pro	Glu	Asp	Cys	
	295					300					305					
CGG	CCC	CGT	GGC	TCA	GTG	AAG	GGG	ACC	GGA	TTG	GAC	TTT	CTA	GAG	GAT	1014
Arg	Pro	Arg	Gly	Ser	Val	Lys	Gly	Thr	Gly	Leu	Asp	Phe	Leu	Glu	Asp	
310					315					320					325	
CCC	AAA	CTC	TGC	TAC	TTG	CTA	GAT	GGA	ATC	CTC	TTC	ATC	TAC	GGA	GTC	1062
Pro	Lys	Leu	Cys	Tyr	Leu	Leu	Asp	Gly	Ile	Leu	Phe	Ile	Tyr	Gly	Val	
				330					335					340		

ATC ATC ACA GCC CTG TAC CTG AGA GCA AAA TTC AGC AGG AGT GCA GAG 1110
Ile Ile Thr Ala Leu Tyr Leu Arg Ala Lys Phe Ser Arg Ser Ala Glu
345 350 355

ACT GCT GCC AAC CTG CAG GAC CCC AAC CAG CTC TAC AAT GAG CTC AAT 1158
Thr Ala Ala Asn Leu Gln Asp Pro Asn Gln Leu Tyr Asn Glu Leu Asn
360 365 370

CTA GGG CGA AGA GAG GAA TAT GAC GTC TTG GAG AAG AAG CGG GCT CGG 1206
Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Glu Lys Lys Arg Ala Arg
375 380 385

GAT CCA GAG ATG GGA GGC AAA CAG CAG AGG AGG AGG AAC CCC CAG GAA 1254
Asp Pro Glu Met Gly Gly Lys Gln Gln Arg Arg Arg Asn Pro Gln Glu
390 395 400 405

GGC GTA TAC AAT GCA CTG CAG AAA GAC AAG ATG GCA GAA GCC TAC AGT 1302
Gly Val Tyr Asn Ala Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
410 415 420

GAG ATC GGC ACA AAA GGC GAG AGG CGG AGA GGC AAG GGG CAC GAT GGC 1350
Glu Ile Gly Thr Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly
425 430 435

CTT TAC CAG GGT CTC AGC ACT GCC ACC AAG GAC ACC TAT GAT GCC CTG 1398
Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
440 445 450

CAT ATG CAG ACC CTG GCC CCT CGC TAACAGCCAG GGCATTTCTC CCTCACGGGC 1452
His Met Gln Thr Leu Ala Pro Arg
455 460

AGATCCCCGG GTACCGAGCT CGAATTC 1479

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 461 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Ala Trp Val Trp Thr Leu Leu Phe Leu Met Ala Ala Ala Lys Val
1 5 10 15

Pro Lys Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro
20 25 30

Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr
35 40 45

Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys
 50 55 60
 Trp Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp
 65 70 75 80
 Asp Phe Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr
 85 90 95
 Ala Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr
 100 105 110
 Phe Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly
 115 120 125
 Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly
 130 135 140
 Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser His
 145 150 155 160
 Lys Phe Leu Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys Lys
 165 170 175
 Ala Ser Gln Asp Val Tyr Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro
 180 185 190
 Gly Gln Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr
 195 200 205
 Gly Val Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr
 210 215 220
 Phe Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys
 225 230 235 240
 Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu
 245 250 255
 Glu Ile Lys Ala Leu Glu Ile Ser Asn Ser Val Met Tyr Phe Ser Ser
 260 265 270
 Val Val Pro Val Leu Gln Lys Val Asn Ser Thr Thr Thr Lys Pro Val
 275 280 285
 Leu Arg Thr Pro Ser Pro Val His Pro Thr Gly Thr Ser Gln Pro Gln
 290 295 300
 Arg Pro Glu Asp Cys Arg Pro Arg Gly Ser Val Lys Gly Thr Gly Leu
 305 310 315 320
 Asp Phe Leu Glu Asp Pro Lys Leu Cys Tyr Leu Leu Asp Gly Ile Leu
 325 330 335

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Phe Ile Tyr Gly Val Ile Ile Thr Ala Leu Tyr Leu Arg Ala Lys Phe
 340 345 350

Ser Arg Ser Ala Glu Thr Ala Ala Asn Leu Gln Asp Pro Asn Gln Leu
 355 360 365

Tyr Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Glu
 370 375 380

Lys Lys Arg Ala Arg Asp Pro Glu Met Gly Gly Lys Gln Gln Arg Arg
 385 390 395 400

Arg Asn Pro Gln Glu Gly Val Tyr Asn Ala Leu Gln Lys Asp Lys Met
 405 410 415

Ala Glu Ala Tyr Ser Glu Ile Gly Thr Lys Gly Glu Arg Arg Arg Gly
 420 425 430

Lys Gly His Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp
 435 440 445

Thr Tyr Asp Ala Leu His Met Gln Thr Leu Ala Pro Arg
 450 455 460

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 443 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
 1 5 10 15

Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn Tyr
 20 25 30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp Met
 35 40 45

Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe
 50 55 60

Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr
 65 70 75 80

Leu	Gln	Ile	Asn	Asn	Leu	Lys	Ser	Glu	Asp	Met	Ala	Thr	Tyr	Phe	Cys	
				85					90					95		
Ala	Arg	Trp	Glu	Val	Tyr	His	Gly	Tyr	Val	Pro	Tyr	Trp	Gly	Gln	Gly	
			100				105				110					
Thr	Thr	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	
		115			120			125								
Ser	Gly	Gly	Gly	Gly	Ser	Asp	Ile	Gln	Leu	Thr	Gln	Ser	His	Lys	Phe	
		130				135			140							
Leu	Ser	Thr	Ser	Val	Gly	Asp	Arg	Val	Ser	Ile	Thr	Cys	Lys	Ala	Ser	
		145				150			155							
Gln	Asp	Val	Tyr	Asn	Ala	Val	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	
				165					170							
Ser	Pro	Lys	Leu	Leu	Ile	Tyr	Ser	Ala	Ser	Ser	Arg	Tyr	Thr	Gly	Val	
			180				185			190						
Pro	Ser	Arg	Phe	Thr	Gly	Ser	Gly	Ser	Gly	Pro	Asp	Phe	Thr	Phe	Thr	
		195				200			205							
Ile	Ser	Ser	Val	Gln	Ala	Glu	Asp	Leu	Ala	Val	Tyr	Phe	Cys	Gln	Gln	
		210				215			220							
His	Phe	Arg	Thr	Pro	Phe	Thr	Phe	Gly	Ser	Gly	Thr	Lys	Leu	Glu	Ile	
		225				230			235							
Lys	Ala	Leu	Glu	Ile	Ser	Asn	Ser	Val	Met	Tyr	Phe	Ser	Ser	Val	Val	
				245					250							
Pro	Val	Leu	Gln	Lys	Val	Asn	Ser	Thr	Thr	Thr	Lys	Pro	Val	Leu	Arg	
			260				265			270						
Thr	Pro	Ser	Pro	Val	His	Pro	Thr	Gly	Thr	Ser	Gln	Pro	Gln	Arg	Pro	
		275				280			285							
Glu	Asp	Cys	Arg	Pro	Arg	Gly	Ser	Val	Lys	Gly	Thr	Gly	Leu	Asp	Phe	
		290				295			300							
Leu	Glu	Asp	Pro	Lys	Leu	Cys	Tyr	Leu	Leu	Asp	Gly	Ile	Leu	Phe	Ile	
		305				310			315							
Tyr	Gly	Val	Ile	Ile	Thr	Ala	Leu	Tyr	Leu	Arg	Ala	Lys	Phe	Ser	Arg	
				325					330							
Ser	Ala	Glu	Thr	Ala	Ala	Asn	Leu	Gln	Asp	Pro	Asn	Gln	Leu	Tyr	Asn	
			340				345			350						
Glu	Leu	Asn	Leu	Gly	Arg	Arg	Glu	Glu	Tyr	Asp	Val	Leu	Glu	Lys	Lys	
		355				360			365							

Arg Ala Arg Asp Pro Glu Met Gly Gly Lys Gln Gln Arg Arg Arg Asn
 370 375 380

Pro Gln Glu Gly Val Tyr Asn Ala Leu Gln Lys Asp Lys Met Ala Glu
 385 390 395 400

Ala Tyr Ser Glu Ile Gly Thr Lys Gly Glu Arg Arg Arg Gly Lys Gly
 405 410 415

His Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr
 420 425 430

Asp Ala Leu His Met Gln Thr Leu Ala Pro Arg
 435 440

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 41 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTGAAAGCTT AGATCTGCCC GTGAGGGAGA AATGCCCTGG C

41

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCGATCTAGA AAGTCCAATC CGGTCCCCTT CACTG

35

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

006793"42496560

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GATCTCTAGA GGATCCCAAA CTCTGCTACT TGC

33

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCGATCTAGA GATCAGCAAC TCGGTGATGT ACTTCAG

37

006190"425650

Claims

1. A bifunctional protein comprising:

- 1) an antigen binding domain derivable from a monoclonal antibody directed against a suitable antigen on a tumor cell,
- 2) a hinge region comprising from about 40 to about 200 amino acids, and
- 3) a functional zeta (ζ) chain derivable from the T-cell antigen receptor (TCR).

2. A DNA encoding a bifunctional protein comprising:

- 1) an antigen binding domain derivable from a monoclonal antibody directed against a suitable antigen on a tumor cell,
- 2) a hinge region comprising from about 40 to about 200 amino acids, and
- 3) a functional zeta (ζ) chain derivable from the T-cell antigen receptor (TCR).

3. A DNA according to claim 2 encoding a protein wherein the antigen binding domain is a single chain antibody, particularly the single chain antibody designated FRP5 (scFv(FRP5)).

4. A DNA according to claim 2 or 3 encoding a protein wherein the hinge region is an immunoglobulin-like hinge region.

5. A DNA according to claims 2 to 4 encoding a protein wherein the functional ζ chain comprises the transmembrane and the cytoplasmic domain.

6. A host cell expressing the DNA of any of claims 2 to 5.

7. A host cell according to claim 6 which is a cytotoxic lymphocyte (CTL).

8. A process for lysing selected tumor cells comprising contacting said tumor cells with CTL producing the protein of claim 1.

9. A process for endowing a CTL with a defined, MHC-independent and MHC-unrestricted tumor cell specificity comprising introducing into said CTL a DNA according to claims 2 to 4.

10. A method for the production of a protein according to claim 1 comprising culturing a

host cell of claim 6 under conditions which allow the expression of a DNA encoding said protein.

11. A composition-of-matter comprising a host cell according to claim 6 or 7.
12. A method of treating cancer comprising the use of a host cell according to claim 7.
13. CTL according to claim 7 for use in a method of treating cancer.
14. Polyclonal or monoclonal antibody specific for a protein according to claim 1.
15. Vector comprising a DNA according to claims 2 to 5

0959674.061900

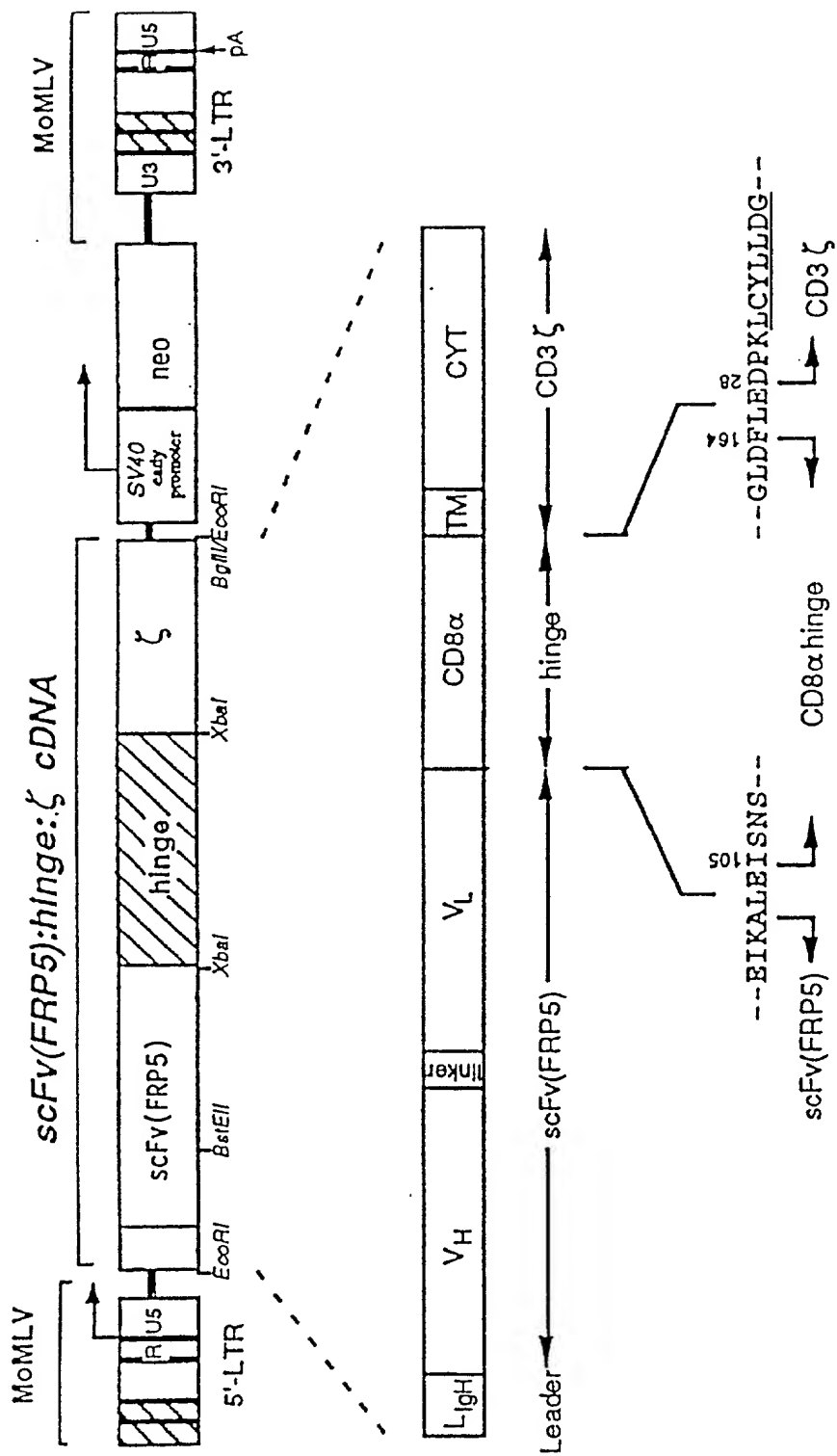
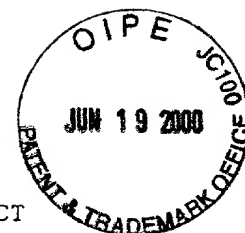


Figure 1 Structure of the pL(FYZ)SN retroviral vector.

DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATIONS



☒ Original ☐ Supplemental ☐ Substitute ☒ PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if more than one name is listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Bifunctional Protein, Preparation and Use

which is described and claimed in:

☐ the attached specification

☐ the specification in U.S. Application No.

filed _____, and as amended on _____ (if applicable).
(day/month/year) (day/month/year)

☒ the specification in PCT International

Application No. PCT/EP 95/01494 filed 20.04.95 ,
(day/month/year)

assigned U.S. Application No. _____ (if applicable), and as amended

☐ under PCT Article 19 on _____ (if applicable)
(day/month/year)

☒ under PCT Article 34 on 13.03.96 (if applicable)
(day/month/year)

☐ and further amended on _____ (if applicable)
(day/month/year)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information which is known by me to be material to the patentability of this application as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119 (a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America relating to this subject matter having a filing date before that of the application on which priority is claimed:

COUNTRY/REGION (OR PCT)	APPLICATION NO.	FILING DATE (day/month/year)	PRIORITY CLAIMED
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European Pat. Off.	94810244.7	02.05.94	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

APPLICATION NO.	FILING DATE (day/month/year)
-----------------	---------------------------------

I hereby claim the benefit under 35 U.S.C. § 120 of any United States applica-
tion(s) or PCT international application(s) designating the United States
listed below and, insofar as the application discloses and claims subject
matter in addition to that disclosed in the prior copending application, I
acknowledge the duty to disclose all information known by me to be material to
patentability as defined in 37 C.F.R. § 1.56 which became available between
the filing date of the prior application and the national or PCT international
filing date of this application:

U.S. APPLICATION No.	FILING DATE (day/month/year)	STATUS		
		<input type="checkbox"/> Patented	<input type="checkbox"/> Pending	<input type="checkbox"/> Abandoned
		<input type="checkbox"/> Patented	<input type="checkbox"/> Pending	<input type="checkbox"/> Abandoned
		<input type="checkbox"/> Patented	<input type="checkbox"/> Pending	<input type="checkbox"/> Abandoned
		<input type="checkbox"/> Patented	<input type="checkbox"/> Pending	<input type="checkbox"/> Abandoned

PCT APPLICATION NO. (designating the U.S.)	INTERNATIONAL FILING DATE (day/month/year)	U.S. APPLICATION No. (if any)	STATUS
			<input type="checkbox"/> Patented
			<input type="checkbox"/> Pending
			<input type="checkbox"/> Abandoned

I hereby appoint the following attorneys and agents, each of them with full power of substitution, revocation and appointment of associates, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole
or first joint inventor

Bernd Groner

Inventor's signature

Bernd Groner

Date

15/10/96
day/month/year

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Citizenship

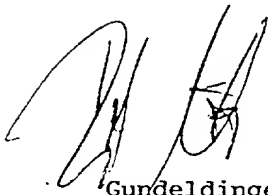
German

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Full name of second
joint inventor, if any

Dirk Moritz

Inventor's signature



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15/10/96
day/month/year

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006T50"42296560
Full name of third
joint inventor, if any

Inventor's signature

Date

day/month/year

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Post Office Address same as above

Full name of fourth
joint inventor, if any

Inventor's signature

Date

day/month/year

Residence

Citizenship

Post Office Address same as above